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14. ABSTRACT

The Tulane/Xavier Biodefense Vaccine Development/Engineering project will develop new vaccines against biological threat agents to aid the war-fighter. Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. One aim of this project will be to compare different nanocarriers for the ability to incorporate biological threat-relevant vaccine antigens and deliver those antigens through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. The use of nanocarriers for vaccine delivery is a platform technology, applicable to delivery of a variety of existing and potential vaccines.

15. SUBJECT TERMS
Vaccines, Bioterrorism, Biothreat, Nanocarrier, Immunity, Prophylaxis

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INTRODUCTION

Infectious diseases remain one of the leading causes of death in adults and children world-wide. Each year, infectious diseases kill more than 17 million people, including 9 million children. In addition to suffering and death, infectious diseases impose an enormous financial burden on society. Although antibiotics and vaccines have been effective at reducing the morbidity and mortality of some infectious diseases, new ones such as AIDS, Lyme disease, West Nile fever, Hanta virus, SARS, and Avian Influenza virus are constantly emerging, while others such as malaria and tuberculosis reemerge in drug-resistant forms. Furthermore, we have an aging adult population with diminishing immune function, increased use of immunosuppressive agents for cancer, tissue transplantation, and autoimmune disease, and an upwardly spiraling cost of health care delivery that makes some existing vaccines unaffordable by the populations at greatest risk. In addition, we now face the possibility of bioterrorism with potentially devastating consequences and a limited number of preventative and therapeutic options.

A great deal of effort has been directed towards developing nonparenteral (needle-free) alternatives to traditional vaccine delivery. Nonparenteral vaccines offer a number of potential advantages over traditional vaccines including 1) the potential to confer mucosal as well as systemic immunity, 2) increased stability, 3) increased shelf-life, 4) elimination of needles and the need for specially trained healthcare specialists to administer vaccines, and 5) potentially lower costs. One such approach, transcutaneous immunization (TCI), is a non-invasive, safe method of delivering antigens directly onto bare skin. Immunization is achieved by direct topical application of a vaccine antigen. Despite the attractiveness of TCI, the technology is limited by the relative inefficiency of transport of large molecular weight vaccine antigens across intact skin.

Recent innovations in transdermal delivery of drugs, including chemical enhancers, electricity, ultrasound, and microneedles, demonstrate the feasibility of large-molecule transport through the skin's permeation-barrier, specifically the stratum corneum. This outer layer of the skin is composed of tightly packed lipid molecules and the dense, crystalline arrangement of these lipids creates the essential barrier to prevent water loss and pathogen entry. Recent evidence has shown that this barrier can be overcome by properly structured nano-sized particles (nanocarriers). This proposal will compare different nanocarriers for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. While the immediate objective will be to deliver vaccines against biological threat agents, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter and civilian populations.

BODY

Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. We will compares different nanocarriers for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum.

In previous funding cycles, we developed the analytical techniques to fully characterize permeation through the skin. We developed cryo-electron microscopy techniques using both cryo-scanning and cryo-transmission. We also fully implemented laser scanning confocal microscopy to understand time resolved permeation through the skin. We also evaluated

ceramide-3 and phosphotidylcholine containing liposomes, nanoscale amphipathic dendrimers, and double emulsions for their ability to deliver a protein antigen across intact skin. In the last funding cycle, we extend those findings on double emulsions, investigated nanoscale unimolecular reverse micelle (URM) carriers, and gel systems that are crystalline mesophases. Finally, we began immunization studies with BSA as a model antigen admixed with ceramide liposomes, water-in-oil-in-water (W1/O/W2) emulsions, tubular liposomes, and silica-tube nanogels. In the current funding cycle, we extended our findings on URM, verifying the utility of these macromolecular carriers in encapsulating polar therapeutics or antigens and compatibilizing them with the non-polar environment of the skin, we developed 3-arm URM carriers that form large aggregates and act to solubilize the polar payload, we developed a method for incorporating ethanol in oil-frozen double-emulsion formulations as a penetration enhancer in a way that stably encapsulates protein antigens, we further defined the hydration parameters for transcutaneous penetration of large biomacromolecules without causing permanent damage to the skin, we incorporated tubular liposomes and their hardened equivalents into novel biopolymer based gels that stay hydrated, thereby increasing the potential for the stratum corneum to allow penetration of the antigen, and we demonstrated stable incorporation of F1-V into ceramide 3 liposomes. Perhaps most importantly, we overcame the technical problem of producing significant amounts of endotoxin-free F1-V for the antigen incorporation, presentation, and immunogenicity studies.

Nanoscale unimolecular reverse micelle (URM) carriers

The goal of these studies was to investigate whether discrete nanoscale unimolecular reverse micelle (URM) carriers can act as efficient carrier of F1-V and enable transport across the skin, without inhibiting the potency of F1-V as an antigen. The predominantly hydrophobic lipids of the stratum corneum act as an effective barrier keeping out most polar compounds, including polar drugs, peptides, proteins, and many potential antigens. Unimolecular reverse micelles offer promise to act as a “Trojan horse” carrier—with a polar core to encapsulate and protect the payload, and a non-polar corona to compatibilize with the skin lipids and allow rapid transcutaneous transport. However, unlike traditional amphiphilic permeation enhancers, micelles, or liposomes, this “micellar” structure is covalent reinforced, and therefore its effectiveness cannot be compromised by leaking contents and disruption due to dilution or changes in polarity.

Initial investigations have relied upon the synthesis of dendrimer initiators with 3, 6, 12, or 24 end groups from which polymer chains can be grown. By polymerizing first a polar monomer, and then a non-polar monomer, a star-polymer can be prepared which has a polar interior, but an oil soluble surface. This approach is particular modular because the number of arms, as well as the length of the polar and non-polar blocks can be tuned to optimize the structure.

In order to verify the utility of these macromolecular carriers in encapsulating polar therapeutics or antigens and compatibilizing them with the non-polar environment of the skin, we investigated the encapsulation of a model, low molecular weight, polar dye in a non-polar solvent, diethyl ether. Acriflavine is a highly polar, water soluble dye with a strong UV absorbance around 460 nm. Due to its polar character, it is expected to be insoluble in the skin lipids as well as non-polar solvents such as hexane or diethyl ether. This was confirmed in a control experiment monitoring the UV absorbance of a saturated solution of acriflavine in diethyl ether, which demonstrates no absorbance because only a negligible amount of polar dye can be dissolved in this lipophilic environment. However, the addition of the 6-arm star-polymer amphiphiles demonstrated a pronounced increasing UV absorbance, proportional to the quantity of carrier, demonstrating that the carrier can encapsulate the polar dye in its polar core, yet maintain its solubility in non-polar solvent because of the lipophilic corona of the carrier (Fig. 1). In an additional control study, this encapsulation ability was compared to a “1-arm” block copolymer that could form self-assembled reverse micelles. While the block copolymer demonstrated some solubilization of the dye, it was significantly less than the same weight of the 6-arm, amphiphilic star nanocarriers (Fig. 2).

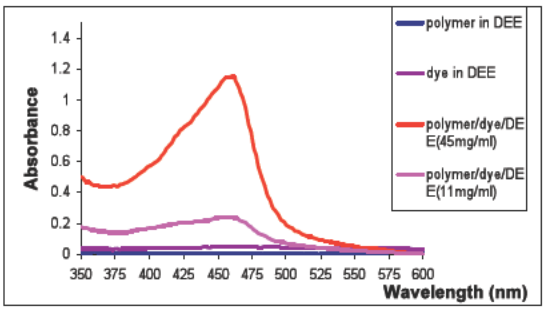


Figure 1: 6-arm star amphiphiles impart lipophilic solubility on polar dye.

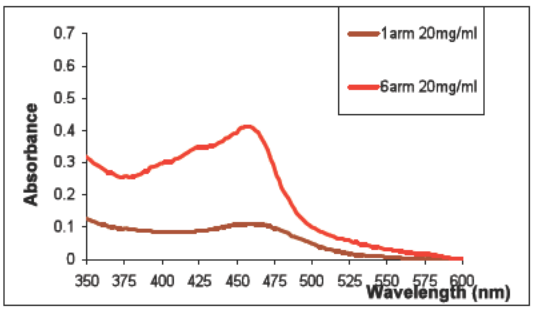


Figure 2: 6-arm star amphiphiles enhance encapsulation of polar guests relative to 1 arm block co-polymer.

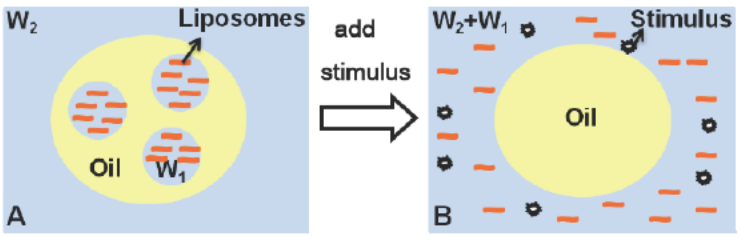
Preliminary fluorescent microscopy studies verified that these nanocarriers significantly enhance the transport of polar small molecules (proflavin dye), which are otherwise insoluble in the lipids of the extracellular matrix, through the stratum corneum. The inherent modularity of this system is expected to make this approach amenable to the encapsulation of significantly larger payloads, such as peptides and proteins, and these are presently under investigation.

Efforts have also focused on thorough synthetic optimization and characterization of these carriers. Light scattering studies show that the 3-arm carriers actually form larger aggregates that act to solubilize the polar payload. The 12-arm carriers on the other hand form smaller aggregates of only a few molecules, because the larger number of branches forces them into a dense packed structure that more closely resembles unimolecular micelles. TEM imaging confirms the sizes of the aggregates observed during light scattering experiments.

In order to more rapidly assemble libraries of carriers, and with the greater number of arms required to make true unimolecular micelles, an alternative approach has been developed whereby preformed amphiphilic block copolymers can be efficiently grafted in a one step "click" coupling reaction using the Huisgen cyclo-addition reaction between a polymer azide and a core molecule with multiple alkynes.

Double Emulsions as Carriers of Proteins for Vaccine Delivery

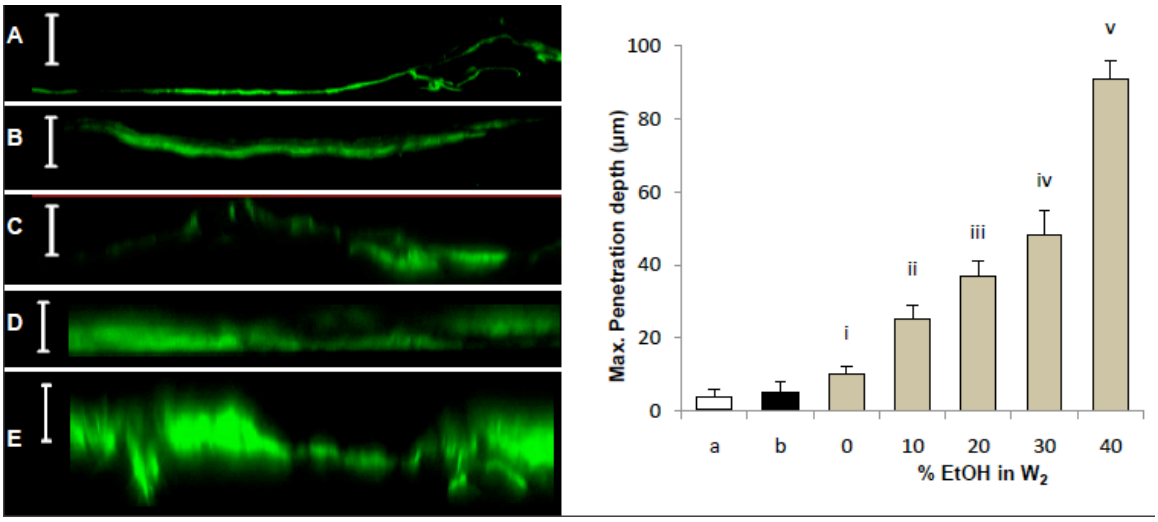
This part of the project consists of sheltering the antigens within the safe micro-environment of the internal droplets of double emulsions for prolonged periods of storage, while at the same time enabling their easy release when applied onto a intact skin. Our studies include the incorporation of liposomes as nanocarriers in the W_1 phase of the microcarrier double emulsion. The hypothesis of that work is that the oil membrane of double emulsions can function as a layer of protection to liposomes and their contents, and thus better control their release (see the schematic below).



The liposomes used were novel tubular liposomes containing a hydrophilic model compound (fluorescein sodium salt, FSS) made by lipids L-α-phosphatidylcholine and Ceramide-VI. Both microscopy-in-capillary experiments, as well as experiments on bulk double emulsions were conducted to determine the effects of double encapsulation on the release of fluorescein from

W_1 phase through the oil (O) phase and into the W_2 phase.

In addition, we examined how ethanol may be incorporated in oil-frozen double-emulsion formulations as a penetration enhancer in a way the encapsulated proteins do not denature. As seen in the following figure, such formulations encapsulating FITC-BSA were able to penetrate up to 91 μm into intact skin, reaching the viable epidermis where the immunocompetent Langerhans cells are located



ELISA studies were performed to examine the effect of the emulsification process and ethanol content on the ability of BSA to form antigen-antibody complexes; results indicated that ethanol content and the emulsification process did not diminish the BSA-antibody complex formation when compared to a BSA standard aqueous solution.

Hydration Effects on Skin Permeability and the Use of Hardened Tubular Liposomes and Novel Gel Phases to Encapsulate Vaccine Antigens

Although hydration is long known to improve the permeability of skin, penetration of large molecules such as proteins is generally limited. Results from this study highlight the need for an extended hydration period before the skin is altered sufficiently for improved protein penetration. In particular, we emphasize the novelty of high resolution electron microscopy that allows us to determine a mechanism for empirical observations in highly hydrated skin. Significant structural alterations, such as swollen corneocytes and separation of lipid bilayers in the stratum corneum intercellular space to form cisternae are observed in skin hydrated for a period of 4 – 10 hours. Networks of spherical particulates, resembling vesicles are observed in cisternae using high resolution cryo-scanning electron microscopy. This is explained in terms of a reverse phase diagram adopted by skin lipids when hydrated, in direct contrast to their transition from flattened unilamellar liposomes in lamellar granules to lipid lamellae between keratinocytes. Confocal microscopy studies show distinct enhancement in penetration of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) through the skin when hydrated for 4 – 10 hours. The fluorescent protein permeates the dermis region of highly hydrated skin, but it mainly accumulates at the surface of native skin. These results point towards the effectiveness of a 4 – 10 hour hydration period to improve the transcutaneous penetration of large biomacromolecules without causing permanent damage to the skin.

In related work, we developed a series of novel tubular and helical liposomes. These liposomes were used as templates to generate highly aspherical silica nanocapsules with length to diameter aspect ratios exceeding 10. Many of these nanocapsules have the morphology of a bulbous end attached to a long tip, mimicking microneedles attached to a reservoir. The fidelity of helical liposomes is transcribed to the silicas and the long tips indicate helically entwined left-handed silica structures. Since shape plays an important role for micro- and nanoscale drug delivery carriers by influencing degradation profiles to release therapeutics and by affecting the targeting ability of the carrier, templating non-spherical liposomes is not only interesting from a materials templating standpoint but the templated non-spherical liposomes may also demonstrate delivery characteristics distinct from their spherical counterparts.

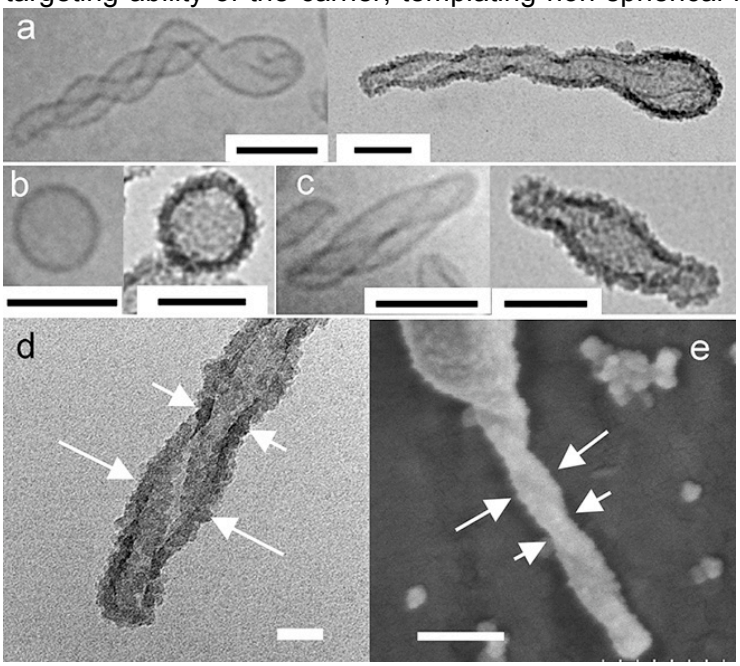
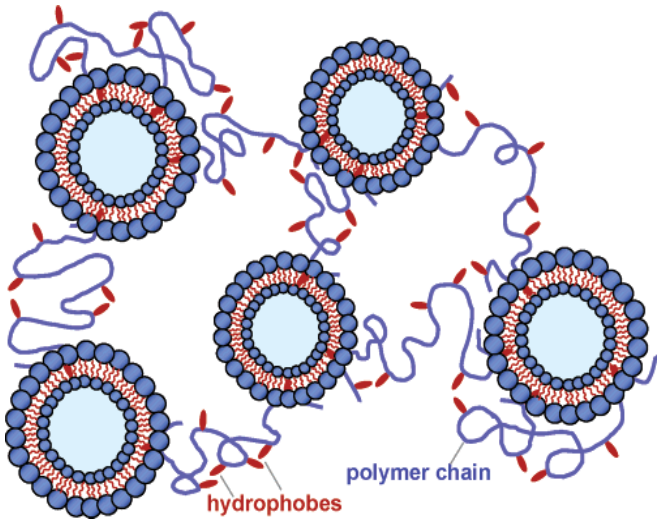


Figure 3. (a) Folded helical, (b) spherical and (c) slight ly elongated liposomes (left) and their silica templated structures (right). Scale bars in (a) to (c) are 100 nm. (d) Close-up TEM image of the tail of a silica templated helical liposome. Scale bar 20 nm. (e) FESEM image showing the topology of a left-handed hollow silica shell. Scale bar 150 nm. Short arrows in (d) and (e) show the narrow section of the entwined liposome tail while the long arrows point towards the wider section of the entwined tails.

In our most recent work, we have incorporated these tubular liposomes and their hardened equivalents into novel biopolymer based gels through collaborations with the University of Maryland (Dr. Srini Raghavan). Through this collaboration, we obtain chitosan functionalized with C12 alkyl groups at random amine positions giving these glucosamine based polysaccharides a hydrophobic component. When these hydrophobically modified chitosans (HMCs) are contacted with lipid vesicles, the hydrophobic groups insert into the bilayers as shown in Figure 4. The vesicles form nodes in a network and the solution forms a gel. When tubular liposomes and their hardened equivalents are incorporated into such gels, they serve as mild abrasives for topical application of antigen. The system stays hydrated, thereby increasing the potential for the stratum corneum to allow penetration of the antigen. Studies incorporating F1-V are underway.



Poly(lactide-co-glycolide) (PLGA) micro/nanocapsules

During the first part of this year, we prepared twenty different formulations of poly(lactide-co-glycolide) (PLGA) micro/nanocapsules containing BSA. However, none of these formulations were useful for the delivery of vaccine through skin because of their relatively larger size. We recently developed five formulations of nanoemulsions containing FITC-BSA with an average particle size of less than 100 nm, which was ideal for permeation through skin.

The nanoemulsion formulation was optimized according to its particle size, viscosity, and temporal stability. The final formulation chosen contained 55% v/v aqueous phase, 40% v/v squalane as the oil phase, and 5% v/v surfactant/co-surfactant blend composed of Tween-80:Span-80 in 1:1 v/v ratio. The permeation profile of each formula is shown in Figure 4 and is summarized in Table 1. As expected, the FITC-BSA solution showed little penetration due to the barrier function of the stratum corneum. As evident in the graph, the nanoemulsion formulation produced the highest drug permeation after 48 hours. Table 1 shows that the nanoemulsion had the highest steady state flux and a higher enhancement ratio than the macroemulsion solution. Additionally, the nanoemulsion produced consistent results compared to the wide distribution observed for the macroemulsion plot. This result is also reflected in the standard deviation of the steady state flux, which is higher for the macroemulsion. The inconsistency of the macroemulsion is due to the wide range of emulsion particle sizes present in the sample, compared to the small particle size and polydispersity index of the nanoemulsion. Thus, the improved permeation of the nanoemulsion FITC-BSA through the skin compared to FITC-BSA solution can be partly attributed to the size of the nanoemulsion.

Table 1. Permeation Parameters of Different Emulsion Formulations Through Mouse Skin

Formulation	Particle Diameter (nm, ± SD)	Polydispersity Index (± SD)	J_{ss} (μg/cm^{2}hr, ± SD)	E_r (relative to FITC-BSA solution)	r^2 of linear portion of permeation plots
Nanoemulsion	144.9 (5.1)	0.207 (0.003)	3.994 (0.223)	36.64	0.9975
Macroemulsion	62.0 (27.8) 105.2 (263.0)[1]	0.256 (0.118)	2.735 (1.767)	25.09	0.9909

[1] bimodal size distribution

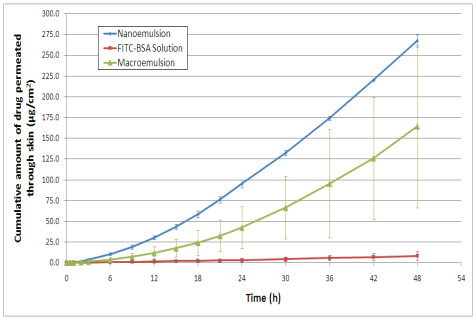


Figure 4. Permeation profile of cumulative amounts of FITC-BSA permeated through mouse skin for different formulations.

Our custom diffusion cell design has allowed us to accurately measure the skin permeability of our optimized nanoemulsion formulation. This study shows that our nanoemulsion formulation has significant potential as a transdermal vaccine adjuvant with 36 times greater skin diffusion compared to FITC-BSA solution. Additionally, the nanoemulsion formulation shows consistent, superior results compared to the larger particle size emulsion, demonstrating the impact of manufacturing technique and particle size on the efficacy of transdermal formulations. Future work will include determining the exact enhancement effect of the individual components of the nanoemulsion formulation to further optimize the skin permeability of the formulation.

F1-V Purification

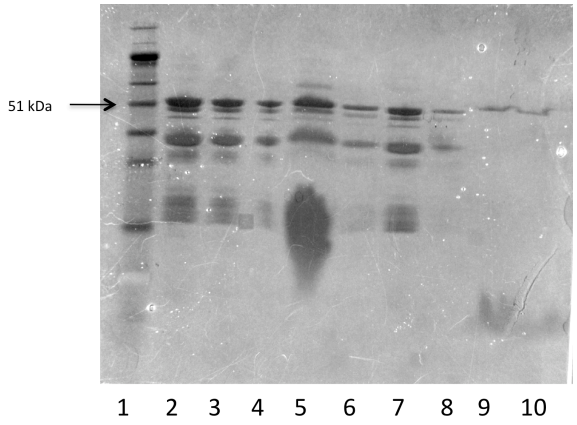
One technical problem we encountered was availability of sufficient quantities of endotoxin free F1-V for the proposed studies. We had obtained the plasmid for production of F1-V from our collaborators at USAMRIID and developed a process for purifying the fusion protein from inclusion bodies in recombinant *E. coli*. However, the yield as insufficient to create a homogenous pool of purified material from which we could remove endotoxin (a confounding variable in immunization studies). Our best production was 5 – 10 mg per batch and we

estimated that we would need a minimum of 150 mg for the incorporation and immunization studies. We inquired about purchasing the material from our collaborators at USAMRIID, but the price we were quoted ($1,500 per 0.5 mg) was not supportable. Consequently, we undertook a process improvement project (changing promoters and production strains, improving purification techniques) and can now make sufficient quantities of purified material for our studies. We would be happy to provide reasonable amounts of endotoxin-free F1-V to any of your awardees free of charge.

F1-V incorporation

We are in the process of incorporating F1-V into each of the nanocarriers in preparation for the immunization studies. In each case, incorporation will first be evaluated *in vitro* prior to immunization. We initiated these studies with ceramide 3 liposomes encapsulating F1-V. First, a high pH buffer suitable for F1-V was used with FITC-labeled BSA to confirm encapsulation of the protein within the liposome vesicles and to determine the effectiveness of ultracentrifugation to concentrate the dispersion. A significant difference in the fluorescence intensity between the supernatant and the pellet containing the liposome vesicles indicated that the vesicles did indeed contain FITC-BSA. Liposome preparations encapsulating F1-V were prepared and the entrapment and antigenicity of the molecule was indicated by SDS-Page and Western blot analysis, as discussed below. Further ultracentrifugation cycles will allow us to more efficiently remove the unencapsulated antigen until the supernatant is free of detectable protein.

The following formulations containing F1-V (molecular weight = 53 kDa) were prepared: ceramide 3 liposomes, squalene nanoemulsions, and water-in-oil-in-water double emulsions. These samples were analyzed with SDS-Page gel and Western blot to determine whether any degradation of the protein occurred during the preparation of the formulations and whether the protein retained its antigenicity (the ability to be recognized by antiserum to F1-V). A representative SDS-page gel with these formulations is shown below:

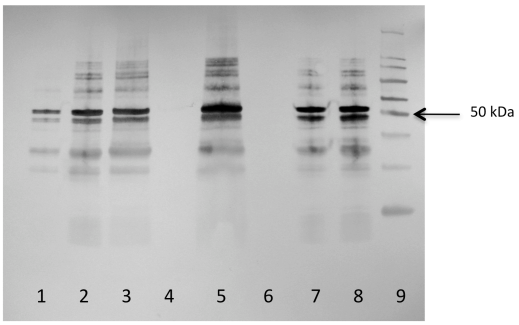


The following components were loaded into each lane:

1) molecular weight marking standard
2) 7.5 µg F1-V in aqueous buffer
3) 3.75 µg F1-V in aqueous buffer
4) 1.5 µg F1-V in aqueous buffer
5) 6.67 µg F1-V encapsulated in water-in-oil-in-water double emulsion formulation
6) 15 µL of liposome pellet collected after ultracentrifugation
7) 3.75 µg F1-V in ceramide 3 liposome (initial preparation before ultracentrifugation) in a total volume of 5 µL
8) 5 µL supernatant after centrifugation of ceramide 3 liposomes
9) F1-V encapsulated in nanoemulsion formulation with protein added prior to emulsification and homogenization
10) F1-V encapsulated in nanoemulsion formulation C with protein added after emulsification and homogenization

From these studies we concluded that 1) There is some slight degradation of the protein once it is lyophilized and resuspended, as indicated by the bands seen at molecular weights less than 53 kDa, the molecular weight of F1-V; 2) None of the preparation/encapsulation techniques resulted in further degradation of F1-V; 3) There is no difference in the different nanoemulsion encapsulation methods as seen in comparing lanes 9 and 10; and 4) There is a significant reduction in the protein concentration between the initial liposome preparation (lane 7) and the supernatant after centrifugation (lane 8), indicating that a significant amount of F1-V is encapsulated in the vesicles (lane 6).

The formulations were then analyzed by Western blot; serum from mice immunized with F1-V was used for staining. A representative membrane is shown below:



The following components were loaded into each lane:

1) 2 µL ceramide 3 liposome supernatant
2) 1.5 µg F1-V in ceramide 3 liposome (initial preparation before ultracentrifugation) in a total volume of 2 µL
3) 15 µL of liposome subnatant collected after ultracentrifugation
4) blank
5) 1.5 µg F1-V encapsulated in water-in-oil-in-water double emulsion formulation
6) blank
7) 0.75 µg F1-V in aqueous buffer
8) 1.5 µg F1-V in aqueous buffer
9) molecular weight marking standard

From these assays we confirmed that encapsulated F1-V protein retains its antigenicity (i.e., is recognized by mouse anti-F1-V sera). This indicates that the antigenicity of the protein is not hindered and that these formulations should be suitable for use in immunization studies to determine their ability to efficiently deliver antigen through intact skin for an appropriate anti-F1-V immune response.

KEY RESEARCH ACCOMPLISHMENTS

- Discrete nanoscale unimolecular reverse micelle (URM) carriers significantly enhance the transport of polar small molecules (proflavin dye) through the stratum corneum, which are otherwise insoluble in the lipids of the extracellular matrix. The inherent modularity of this system is expected to make this approach amenable to the encapsulation of significantly larger payloads, such as peptides and proteins, and these are presently under investigation.

- 3-arm URM carriers actually form larger aggregates that act to solubilize the polar payload. The 12-arm carriers on the other hand form smaller aggregates of only a few molecules, because the larger number of branches forces them into a dense packed structure that more closely resembles unimolecular micelles.

- Ethanol can be incorporated in oil-frozen double-emulsion formulations as a penetration enhancer in a way the encapsulated proteins do not denature.

- A 4 – 10 hour hydration period to improve the transcutaneous penetration of large biomacromolecules without causing permanent damage to the skin.

- We have incorporated tubular liposomes and their hardened equivalents into novel biopolymer based gels that stay hydrated, thereby increasing the potential for the stratum corneum to allow penetration of the antigen.

- Process improvement for production of significant quantities of endotoxin-free F1-V.

REPORTABLE OUTCOMES

G. Tan, P. Xu, V. T. John, L. B. Lawson, L. C. Freytag, J. D. Clements, and J. He. “Mechanistic insights into stratum corneum hydration and implications for transcutaneous vaccine antigen penetration.” CRS Newsletter. 26(1):10-12.

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CONSLUSIONS

Encapsulating a vaccine antigen within or adsorbing it to appropriate nanocarriers should facilitate transport through the stratum corneum to the targeted dendritic cells of the epidermis and dermis to initiate an immune response. Tailoring the nanocarriers to optimize encapsulation and/or adsorption and permeation efficiency requires an understanding of the interactions between the molecules composing the carrier, the antigen of interest, and the skin components in addition to the potential immune response to the antigen and the possible effect of the carrier or coadministered adjuvants on this response. Antigen-presenting cells show more efficient uptake of antigen incorporated into or onto a vesicular or particulate carrier, suggesting the potential for nanocarriers to enhance not only transport of the antigen through the skin’s barrier but also uptake of the antigen once it reaches the dendritic cells of the viable epidermis and dermis. Nanocarrier-based transcutaneous vaccines represent a promising application of nanotechnology for delivery of vaccines against biological threat agents. Moreover, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter.